Exhibit A

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Creation of Novel Protein Transduction Domain (PTD) Mutants by a Phage Display-Based High-Throughput Screening System

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Significant research effort is currently focused on Protein Transduction Domains (PTDs) as potential intracellular drug delivery carriers. However, the application of this technology is limited because the transduction efficiencies are often insufficient for therapeutic purposes, even using HIV-1 Tat peptide. Here we describe a high-throughput screening method based on a phage display system for isolating novel PTDs with improved cell penetration activity. The screening method involves using protein synthesis inhibitory factor (PSIF) as cargo of PTD. Using this method, several Tat-PTD mutants of superior cell-penetrating activity were isolated. Interestingly, the amino acid sequence of the PTD mutants contained some characteristic residues, such as proline. Thus, our screening method may prove useful in determining the relationship between protein transduction and amino acid sequence.

Key words phage display system; protein transduction domain; high-throughput screening; HIV-1 Tat; intracellular drug delivery

Recent advances in proteomics have allowed a number of refractory diseases, such as cancer and neurodegenerative disorders, to be studied at the molecular level. The main causative factor of such disease states is often associated with intracellular organelles or particular subcellular proteins. Thus, the intracellular organelles, proteins or genes might constitute the therapeutic target. Recently, it was discovered that certain peptides, referred to as protein transduction domains (PTDs), can penetrate cells accompanied by a large molecular cargo. Considerable research effort is currently focused on utilizing PTDs as peptide-based carriers for intracellular drug delivery.[1—3]

Tat peptide, derived from the HIV-1, and Antennapedia peptide, derived from Drosophila Antennapedia homeotic transcription factor, are well known PTDs that have been tested as drug delivery carriers for various disease models.[4—9] PTDs can even deliver bulky molecular cargos (>100 kDa) into a wide variety of cell types.[10—13] However, to use PTDs as effective intracellular drug delivery carriers with clinical applications, it is necessary to create novel PTDs with greater protein-transduction potency than exists naturally.

An attempt to create a novel PTD by modification of the peptide structure has already been reported.[14,15] However, because it is difficult to predict the transduction activity of the peptide based on structural information alone, novel peptides must be generated by introducing amino acid substitutions and then the effects determined by trial and error. Recently, we have successfully generated a technology for creating novel muteins (mutant proteins) that have non-native functions using a phage display system.[16] This prompted us to apply phage display technology to screen for novel PTDs.

The phage display system is a protein selection methodology in which a library of mutant proteins or peptides can be screened and the desired molecules easily identified by linking DNA information (genotype) with phenotype (protein expression).[16—20] By applying this methodology, novel PTDs can be selected such as those transduced into the cell by a different mechanism or those with tissue/cell specificity. In general, the phage display system is used to isolate antibody and peptide ligands using an affinity selection step to target the desired molecules. However, for the discovery of PTDs it is necessary to construct a screening method to select clones that are transduced into the cell rather than simply selecting those that bind to the cell surface. We designed a high-throughput screening method to isolate effective PTDs by fusing PTD with Protein Synthesis Inhibitory Factor (PSIF).[21] Here, we used our methodology to identify novel Tat mutants with greater transduction potency than wild-type Tat PTD.

MATERIALS AND METHODS

Library Construction A gene library of Tat mutant peptides was constructed by randomization of codons (except arginine codons) of Tat [47—57] using PCR primers containing NNS sequences (N; A/T/G/C, S; G/C). Two primer sequences were used in this PCR. Forward primer, Y-oligo22 3′ ex (5′-TCA CAC AGG AAA CAG CTA TGA CCA TGA TTA CGC CAA GCT TTG GAG CC-3′) contained a *Hind*III site and annealed on pCANTAB phagemid vector. Reverse primer, Tat[47—57] R (5′-TC ATC CTT GTA GTC TGC GGC CGC ACG ACG ACG SNN ACG ACG SNN SNN ACG SNN SNN GGC CAT GGC CGG CTG GGC CGC ATA GA AAG-3′) contained five NNS codons and a *Not*I site. After amplification of the Tat[47—57] mutant gene, the PCR fragments were digested with *Hind*III and *Not*I and cloned into the pCANTAB phagemid vector (Invitrogen Corp., Carlsbad, CA, U.S.A.). *E. coli* TG1 cells (Stratagene, La Jolla, CA, U.S.A.) were transformed with the phagemid by electroporation and then phage displaying Tat mutant peptide library were produced by infection of M13KO7 helper phage (Invitrogen Corp.).

Cell Panning The human keratinocyte cell line, HaCaT, was seeded in 6 well tissue culture plates at 5×10^5 cells/well

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and cultured overnight. The culture medium was changed to Opti-Mem I medium (Invitrogen Corp.) containing 2% BSA for blocking and incubated for 2 h at 37 °C. Purified phage library was pre-incubated with the same medium at 4 °C for 1 h. The phage solution was then applied to the HaCaT cells and incubated for 2 h at 37 °C. Unbound phage was removed by extensive washing (20×) with PBS (pH 7.2). Phage particles bound or internalized with the HaCaT cells were subsequently rescued by adding ice cold 50 mM HCl to each well and incubating for 10 min at 4 °C. The solution containing lysed cells and phage library was collected and neutralized by adding 1.0 M Tris–HCl pH 8.0. The phage clones contained in the solution were propagated by infecting *E. coli* TG1 and applied for the next round of panning. The cell panning was repeated two more times (*i.e.* 3 panning rounds in total).

Expression of PTD-PSIF Proteins Protein synthesis inhibitory factor (PSIF, PE fragment) is an approximately 40 kDa fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa*[22] (ATCC strain No.29260). PSIF lacks its cell binding domain, and has been successfully used as a cytotoxic portion of a recombinant immunotoxin.[23] We cloned the cDNA for PSIF from *Pseudomonas aeruginosa*, Migula by PCR using the primer set: 5′-GAT GAT CGA TCG CGG CCG CAG GTG CGC CGG TGC CGT ATC CGG ATC CGC TGG AAC CGC GTG CCG CAG ACT ACA AAG ACG ACG ACG ACA AAC CCG AGG GCG GCA GCC TGG CCG CGC TGA CC-3′ and 5′-GAT CGA TCG ATC ACT AGT CTA CAG TTC GTC TTT CTT CAG GTC CTC GCG CGG CGG TTT GCC GGG-3′. The PCR product was cloned into modified pCANTAB phagemid vector. After 3 rounds of cell panning, the enriched library of PTD candidate cDNA clones were purified from phage-infected TG1 cells and inserted into the PSIF-fusion expression vector derived from phagemid pCANTAB5E. TG1 cells were transformed with the PTD-PSIF fusion library and monocloned. Transformed TG1 clones were picked, transferred to a 96 well plate format and cultured in 2-YT medium (Invitrogen Corp.) containing 2% glucose and 100 μg/ml ampicillin until the OD_{600} reached 0.5. PTD-PSIF protein was expressed in the supernatant by culturing the cells for 12 h at 37 °C in 2-YT growth medium with no glucose in the presence of 100 mM IPTG. These supernatants were harvested and used for the cellular cytotoxicity assay.

Cytotoxicity Assay of PTD-PSIF Fusion Protein against HaCaT Cells HaCaT cells were seeded on 96 well tissue culture plates at 1.5×10^4 cells/well in Opti-Mem I medium containing 50 μg/ml cycloheximide. Each culture supernatant from the PTD-PSIF clones was then added to an individual well. After incubation at 37 °C for 24 h, viability of HaCaT cells was assessed using the MTT assay.

Flow Cytometry Analysis of FITC-Labeled PTDs on Live Cells HaCaT cells were seeded on 24 well tissue culture plates at 1.0×10^5 cells/well. After incubation for 24 h at 37 °C, the cell monolayer was treated with FITC-labeled PTDs diluted in growth medium at a final concentration 10 μM for 3 h. Cells were then washed and any PTDs adsorbed to the cell surface digested using 2.5% trypsin. Cellular fluorescence was then measured by flow cytometry (Becton Dickinson, Oxford, U.K.).

***In Vitro* Safety Assessment** HaCaT cells were seeded on 96 well tissue culture plates at 1.6×10^4 cells/well. After incubation for 24 h at 37 °C, FITC-labeled PTDs were added to the cell monolayer at three different concentrations (3 μM, 10 μM or 30 μM). After additional incubation for 24 h at 37 °C, cell viability was assessed by the WST-8 assay (Dojindo Lab., Kumamoto, Japan).

Fluorescence Microscopic Analysis HeLa cells were seeded on a chamber coverglass at 3.0×10^4 cells/well in culture medium (MEM 10% fetal calf serum) and incubated for 24 h. A 2 μM aliquot of streptavidin modified Qdot525 (Quantum Dot Co., Hayward, CA U.S.A.) was incubated with 200 μM of synthesized biotinylated PTDs at room temperature for 5 min and diluted in culture medium containing 10% fetal calf serum (FCS) and 5 nM PTD-conjugated Qdot. HeLa cells were then treated with the culture medium containing PTD-Qdot and 100 ng/ml Hoechst 33342 (Invitrogen Corp.) and incubated at 37 °C for 1 h. The medium was then changed for Qdot-free medium and the cells observed by fluorescence microscopy using an Olympus IX-81 microscope (Olympus Co., Tokyo, Japan) at various time points.

RESULTS AND DISCUSSION

In this study, a screening method for Tat PTD mutants with efficient cell penetrating activity was established and novel peptide sequences were identified (Fig. 1). Mutagenic PCR, using primers Y-oligo22 3′ex and Tat[47—57]R), was used to prepare a mutant peptide gene library of Tat in which 5 codons were randomized within the Tat[47—57] peptide. All the natural arginine codons of this peptide were retained because arginine was reported to have an important roll for penetrating into the cells.[24] The PCR product was then ligated into the phagemid vector. Approximately 16 million colony forming units (cfu) were obtained after transformation of *E. coli* TG1 with the phagemid. DNA sequence analysis of the library confirmed it to be derived from independent clones (Table I). Our results established that the library had an enormous repertoire covering the 3.2 million theoretical combinations of 5 amino acids. From this, a 1.0×10^{12}—10^{13} cfu phage library displaying Tat mutant was prepared. In order to enrich the phage clones which bound or internalized to the cells, 3 rounds of cell panning using the HaCaT cell line was performed. The enriched phage clones included not only PTDs capable of penetrating the cell but also those peptides which simply bind to the cell surface.

To allow the differential selection of PTDs capable of penetrating the cell we designed a high-throughput screening method by fusing PTD with PSIF. PSIF from *Pseudomonas aeruginosa*, was not by itself cytotoxic because the cell-binding domain was truncated. However, PSIF shows cytotoxicity when it is fused to a carrier, such as PTD, because it can then enter the cytoplasm.[21] PSIF-fusion is a simple and effective screening method for novel PTDs because the penetrating ability of the peptide can be evaluated from the cytotoxic effects of the fused protein. Figure 2A shows the cellular uptake of PTD-PSIF fusion from the Tat mutant library before cell panning. No clones displaying stronger cytotoxicity than wild-type Tat-PSIF fusion could be detected. However, after 3 rounds of cell panning, over 80% of the analyzed 800 clones showed stronger cytotoxicity than wild-type Tat-PSIF.

(Fig. 2B). Using this rapid PSIF screening method, we isolated superior PTD candidates in only 2—3 weeks. Clones showing enhanced cytotoxicity over wild-type Tat peptide were isolated and the DNA sequences analyzed.

Next, FITC labeled PTD mutant candidates were synthesized and cellular uptake was determined by flow cytometry (Fig. 3). Each of the PTD candidates displayed similar or increased uptake compared with wild-type Tat[47—57] or Tat[48—60]. In particular, cellular uptake efficiency of YM2 or YM3 was 2.5 to 3 fold greater than wild-type Tat. Table 2 shows the amino acid sequences of clones YM2 and YM3. Some clones, including YM2 or YM3, have an increased number of arginine residues (clones 1, 6 and 7, Table 1). Moreover, all the clones shown in Tables 1 and 2 have almost the same isoelectric point (pI) of *ca* 13. In general the transduction ability of PTDs is associated with cationic amino acid residues, such as arginine. However, our data indicates that the transduction ability of PTDs is not wholly dependent on the total number of arginine residues or the overall pI. Interestingly, YM2 and YM3 include some characteristic amino acid residues, such as proline. In addition, these PTD candidates have arginine at the same position as Tat 54, which is thought to be important for transduction. In this way, our phage display system can correlate the amino acid sequence of the peptides with transduction ability. Thus, for the first time, it may be possible to experimentally determine the factors that influence intracellular transduction other than cationic amino acids or pI.

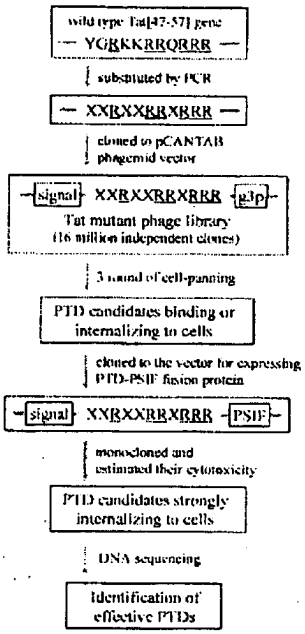

Fig. 1. Overview of the Creation of the Novel PTDs Using a Phage Display System Based High-Throughput Screening Method

The Tat mutant peptide gene library was constructed by randomization of the Tat[47—57] sequence, except for the arginine codons. Fixed arginine residues are underlined. Substituted amino acids are shown as X. After amplification, mutant Tat genes were cloned into pCANTAB phagemid vector. The Tat mutant phage library was produced from phagemid transformed TG1. The phage library was then subjected to 3 rounds of cell panning as described in Materials and Methods. Mutant Tat clones binding or internalizing to the cell were initially concentrated from the library. PTD candidates were then purified and cloned into PSIF expression vector. Monoclonal TG1s containing individual PTD-PSIF encoding phagemid were picked up separately into a 96 well format. The cytotoxicity of the PTD-PSIF proteins was assessed in order to isolate Tat peptide mutants that are strongly internalized within the cell. Approximately 1000 clones can be simultaneously assayed for cytotoxicity by this procedure. The amino acid sequence of effective Tat mutants were readily obtained from their DNA sequence.

To utilize PTD as an effective intracellular drug delivery carrier, the peptide must be nontoxic to the cells. Using the assay for HaCaT cells, no cytotoxicity was observed with peptides YM1, YM2 and YM3 (Fig. 4). Polyarginine is one of the representative artificial PTDs and, like Tat peptide, is highly efficient at transducing cargo into the cell.[24] However, polyarginine (Arg 11) displayed more cytotoxicity than Tat peptide.[25] Our initial assessment, conducted on a specific cell line, indicates that all 3 novel PTDs are safe drug carriers.

Another research group has also reported the generation of novel PTDs with enhanced transduction potential compared to that of Tat peptide.[14] However, it was never demonstrated whether these PTDs actually introduced cargos into the cell. Therefore, we examined whether our PTDs candidates were able to deliver macromolecules. Qdots, a fluorescent semiconductor nanocrystal, was used as a model macro drug molecule. Qdots streptavidin conjugate was modified with either biotinylated Tat[47—57] or YM3 peptide and then applied to cultured HeLa cells. After 1 h, Tat[47—57] or YM3 labeled Qdot were localized near the cell membrane (Figs. 5a, d). For these observations, Hela cell was used in spite of HaCaT cell because the localization analysis of Qdots in HaCaT cell was difficult due to its small cytoplasmic area. Upon further incubation, the location of the Qdot-PTD conjugates changed (Figs. 5b, e) until after 20 h the Qdot was observed at the perinuclear region (Figs. 5c, f). However, Qdots was not ob-

Table 1. Amino Acid Sequences and pI Values of Tat Mutants from the Library

Clone	Position											pI
	47	48	49	50	51	52	53	54	55	56	57	
Tat[47—57]	Y	G	R	K	K	R	R	Q	R	R	R	12.8
Clone1	T	L	R	T	R	R	R	N	R	R	R	13.3
Clone2	N	Y	R	T	G	R	R	K	R	R	R	12.8
Clone3	L	T	R	Q	T	R	R	M	R	R	R	13.2
Clone4	S	K	R	T	W	R	R	N	R	R	R	13.2
Clone5	K	E	R	H	L	R	R	H	R	R	R	12.8
Clone6	D	R	R	N	S	R	R	N	R	K	R	12.9
Clone7	H	R	R	P	V	R	R	F	R	R	R	13.3
Clone8	A	P	R	D	W	R	R	A	R	R	R	12.8

Sequence analysis of random phage clones isolated from the library. The library confirmed it to be derived from independent clones.

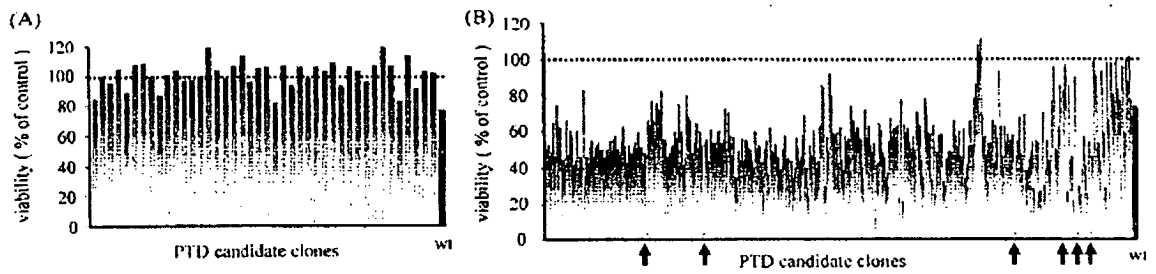

Fig. 2. Cellular Cytotoxicity Assay of the Monoclonal PTD Candidate-PSIF Fusion Proteins to HaCaT Cells

These figures show the cellular uptake of individual clones-PSIF fusion proteins from (A) Tat mutant library before cell panning and (B) concentrated novel PTD candidates after 3 rounds of cell panning. Cellular cytotoxicity was assessed using the MTT assay. The dose of PTD-PSIF fusion clones was adjusted to retain *ca.* 80% viability when using wild-type Tat-PSIF fusion protein (left stripy column). Clones in the arrowed columns showed greater cytotoxicity over wild-type Tat-PSIF fusion protein.

Table 2. Nucleotide and Amino Acid Sequences and pI Values of Novel PTDs

Clone	Position											pI
	47	48	49	50	51	52	53	54	55	56	57	
Tat[47--57]	Y	G	R	K	K	R	R	Q	R	R	R	12.8
	TAC	GGT	CGT	AAA	AAA	CGT	CGT	CAG	CGT	CGT	CGT	
YM1	R	N	R	A	R	R	R	Q	R	R	R	13.4
	AGG	AAC	CGT	GCC	CGC	CGT	CGT	CAG	CGT	CGT	CGT	
YM2	P	V	R	R	P	R	R	R	R	R	R	13.4
	CCC	GTG	CGT	CGC	CCC	CGT	CGT	CGG	CGT	CGT	CGT	
YM3	T	H	R	L	P	R	R	R	R	R	R	13.3
	ACC	CAC	CGT	TTG	CCC	CGT	CGT	CGC	CGT	CGT	CGT	

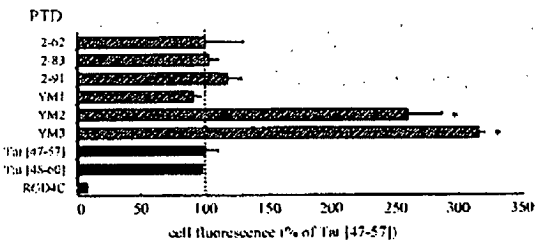

Fig. 3. Cellular Uptake of PTD-FITC Conjugates into HaCaT Cells

FITC labeled PTDs were incubated with HaCaT cell monolayer for 3 h and their cellular uptakes were estimated by flow cytometry analysis. Stripy columns show uptakes of PTD candidates from the Tat mutant library. Black columns show uptake of control PTDs. Control PTD sequences are as follows: Tat[47—57] (wild type Tat PTD): YGRKKRRQRRR, Tat[48—60]: GRKKRRQRRRPPQ, RGD4C: CDCRGDCFC. This experiment was performed at $n=3$. Each data value represents the mean±S.D. * $p<0.005$, compared with Tat[47--57].

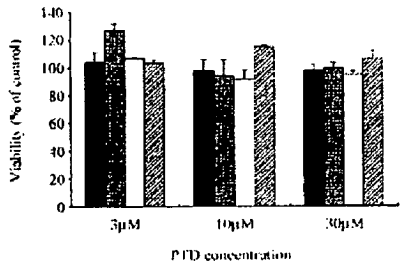

Fig. 4. *In Vitro* Safety Assessment of Tat Mutants

FITC labeled Tat[47--57] (■), YM1 (⊞), YM2 (□) or YM3 (■) were incubated with HaCaT cell monolayer for 24 h and their cytotoxicity was estimated using the MTT assay. Non-treated cells were arbitrarily given a value of 100%.

served in the cell nucleus. Recently, Tat peptides were reported to enter the cell by macropinocytosis.[26,27] By analogy, a large proportion of the incorporated Qdots may become trapped in the macropinosome and thus fail to transfer into the nucleus. Therefore, to achieve efficient drug delivery into the cytosol or organelles, the cargo must be released from the macropinosome. One possible strategy would be to incorporate the HA2 peptide to enhance the liberation of carrier and cargo protein from the endosome.[26,28]

It is reported that PTDs are able to deliver various bioactive molecules into cells. However their transduction efficiencies are not sufficient to achieve effective protein-based therapy. In this report, we used a high throughput screening method to successfully identify novel PTD mutants with improved cell penetrating activity over wild-type Tat peptide. The PTD mutants were found to contain some characteristic amino acids. These findings indicate that there may be many factors to account for cell penetration other than the presence of cationic amino acids. Using our high-throughput screening method, it should be possible to formulate some generic rules concerning the mechanism of cell penetration and subcellular transport. In conclusion, our high-throughput screening system is expected to contribute to the development of protein-based therapies.

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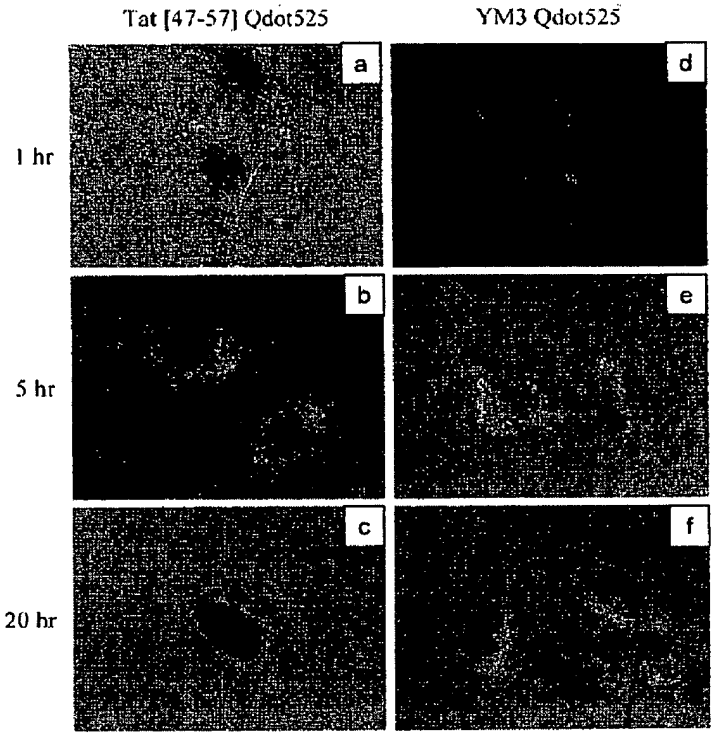

Fig. 5. Cellular Uptake and Intracellular Behavior of PTD-Qdot Complex in HeLa Cells

5 nM Tat[47—57] (a, b and c) or YM3 (d, e and f) labeled Qdots were incubated with Hela cells. The cells were observed using fluorescence microscopy after 1 h (a and d), 5 h (b and e) or 20 h (c and f). The cell nucleus was stained with Hoechst 33342.

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Exhibit B

"protein transduction domain" - PubMed Results - Microsoft Internet Explorer

파일(F) 편집(E) 보기(V) 즐겨찾기(A) 도구(T) 도움말(H)

뒤로 검색 즐겨찾기

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1: Meade BR, Dowdy SF. Related Articles, Links

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Adv Drug Deliv Rev. 2007 Oct 22; [Epub ahead of print]
PMID: 18155315 [PubMed - as supplied by publisher]

2: Koya V, Shun L, Sun YP, Purich DL, Atkinson MA, Li SW, Yang LJ. Related Articles, Links

Reversal of Streptozotocin-Induced Diabetes in Mice by Cellular Transduction with Recombinant Pancreatic Transcription Factor Pdx1 - A Novel Protein Transduction Domain Based Therapy.
Diabetes. 2007 Dec 17; [Epub ahead of print]
PMID: 18086901 [PubMed - as supplied by publisher]

3: Adachi T, Hanaka S, Masuda T, Yoshihara H, Nagase H, Ohta K. Related Articles, Links

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Exhibit C

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Items 1 - 20 of 707 Page 1 of 36 Next

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Exhibit D

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Page 1 of 6 Next

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